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Antibodies to Glycolipids Activate Complement and Promote Proteinuria in Passive Heymann Nephritis

Martin Susani,* Matthias Schulze,† Markus Exner,* and Dontscho Kerjaschki*

From the Division of Ultrastructural Pathology and Cell Biology, Institute of Clinical Pathology, University of Vienna, Vienna, Austria; and Division of Nephrology,† Department of Internal Medicine, Medizinische Hochschule Hannover, Hannover, Germany*

Passive Heymann nephritis is an experimental rat model of human membranous nephropathy induced by injection of antisera against crude renal cortical fractions such as Fx1A or rat tubular microvilli. This results in the formation of subepithelial immune deposits, the activation of the C5b-9 membrane attack complex of complement, and severe proteinuria. While the formation of immune deposits is attributed to* in situ *immune complex formation with antibodies specific for the gp330-Heymann nephritis antigenic complex (HNAC), activation of complement and proteinuria appear to be caused by at least one additional antibody species present in anti-Fx1A sera. We have separated by affinity absorption polyspecific antisera against Fx1A and rat microvilli into one IgG fraction directed specifically against microvillar proteins (anti-Fx1A-prot) and another IgG fraction specific for glycolipids (ant-Fx1A-lip) of tubular microvilli. When injected into rats, the anti-Fx1A-prot fraction induced immune deposits but failed to activate complement or produce proteinuria, similar to results obtained with affinity-purified anti-gp330 IgG. When the antibodies of the anti-Fx1A-lip fraction were injected alone they did not bind to glomeruli. By contrast, when the IgGs specific for the Fx1A-prot fraction (or for gp330-HNAC) were combined with those directed against the Fx1A-lip glycolipid preparation, immune deposits were formed,* in situ *complement activation was observed, and also proteinuria was induced. It is concluded that within anti-Fx1A and anti-microvillar sera there are at least two IgG fractions of relevance for the development of PHN: one directed against the gp330-HNAC complex which is responsible for the development of immune deposits, and a second specific for glycolipid antigen(s) which activate(s) the complement cascade. (Am J Pathol 1994, 144:807–819)

Active Heymann nephritis (HN) is an experimental rat model of membranous nephropathy that closely resembles the human disease in its principal features: the formation of subepithelial immune deposits in renal glomeruli and proteinuria.[1,2] Passive Heymann nephritis (PHN) is a variant in which these features are rapidly produced by injection of heterologous antibodies against crude extracts of kidney cortex, called Fx1A,[3] or a glycolipoprotein fraction prepared from it (RTEα5),[3] or isolated microvillar fractions (MV) from proximal tubules of rat kidney.[4] These polyspecific antisera contain, among several other specificities, IgG directed against a large membrane glycoprotein complex, the gp330-Heymann nephritic complex (HNAC),[5,6] which is present in glomerular epithelial cells.[7] The HNAC is assembled of the large glycoprotein gp330 and an associated 44-kd protein (called C14[5] or receptor-associated protein, RAP)[6] and serves as antigenic target for the formation of the initial immune complexes *in situ* in clathrin-coated pits of the cell membranes on the "soles" of the podocytes. Recently, a nephritogenic epitope on the 44-kd component of the HNAC was narrowed down to ~80 amino acids.[6] By contrast, much less is known about the molecular aspects of capillary wall damage and proteinuria in PHN. It is now established that the local

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Address reprint requests to Dr. D. Kerjaschki, Institute of Clinical Pathology, University of Vienna, Allgemeines Krankenhaus, Währinger Gürtel 18–20, 1090 Vienna, Austria.

activation of C5b-9 within the immune deposits is essential for the development of proteinuria,[8–10] but how this activation is mediated is unknown. In this study we have identified an IgG species in the polyspecific anti-Fx1A and the anti-MV sera that is directed against glycolipid antigen(s) and which is involved in the activation of complement to the C5b-9 membrane attack complex within the immune deposits in PHN.

Materials and Methods

Animals

Male Sprague-Dawley rats (150 to 200 g) were obtained from the Tierzuchtinstitut der Universität Wien, Himberg. The use of rats for experimental purposes was permitted by the Austrian Ministry of Science.

Preparation of Antibodies

Sheep anti-Fx1A serum was obtained from Dr. W. G. Couser (Seattle, WA) and was prepared as described.[3] Rabbit antibodies to isolated tubular microvilli from rat kidneys[11] were produced by intradermal immunization followed by two boosts, as described.[7] IgG from both sera were purified on a protein G-agarose column (Pharmacia, LKB, Uppsala, Sweden).[12] Affinity-purified rabbit anti-dipeptidyl peptidase (DPP IV) IgG was a gift from Dr. P. Verroust. Rabbit anti-human laminin serum was from BRL (Gaithersburg, MD). The concentration of IgG was determined by reading of the OD_{280}.

Monospecific anti-gp330 IgG was produced by circulating of sheep Fx1A IgG over a CNBr-Sepharose 4B column to which 500 μg of gp330 were bound, which was purified by electroelution from preparative SDS-PAGE as described.[7] IgG was eluted with 100 mmol/L glycine buffer, pH 2.7, neutralized immediately with 1 mol/L Tris-HCl buffer, pH 8.0, dialyzed against PBS, and concentrated in Aquacide II (Calbiochem, La Jolla, CA). Fx1A minus anti-gp330 IgG was prepared by repeated absorption of anti-Fx1A IgG on the gp330-affinity column until the unbound IgG was found to be completely depleted of its anti-gp330 specificity by immunoblotting. The fractionation procedure is shown in Figure 1.

Preparation of Glycolipid-Specific IgG Fractions (Fx1A-lip IgG)

Sheep anti-Fx1A IgG and rabbit anti-MV IgG were adsorbed to liposome suspensions which were prepared from isolated tubular microvilli of 20 rat kidneys.[11] Stepwise extraction of lipids was performed at 4 C with chloroform/methanol 2:1, 1:1, 1:9, and with methanol alone, and sonicated.[13] The extracted lipids in the pooled organic solvents were dried in a rotary evaporator, dissolved in chloroform, centrifuged for 15 minutes at 20,000 rpm, and dried again. Liposomes were formed by addition of 1 mol/L phosphate buffer, pH 7.2, at 37 C, by shaking, followed by incubation at 20 C for 2 hours and ultrasonication for 5 minutes (Branson, CT) after the first hour. The liposomes were centrifuged at 17,000 × *g* and washed twice by suspension in PBS.[14] Twenty mg of sheep anti-Fx1A IgG or rabbit anti-MV IgG in PBS were added to the liposome suspension, causing immediate agglutination, followed by incubation for one hour at 4 C. The agglutinated liposomes were pelleted, resuspended in 100 mmol/L triethylamine, pH 11.5 at 4 C, for elution of the bound IgG[15] and immediately centrifuged at 40,000 × *g* for 15 minutes in a Sorvall OM2 ultracentrifuge. The supernatant was

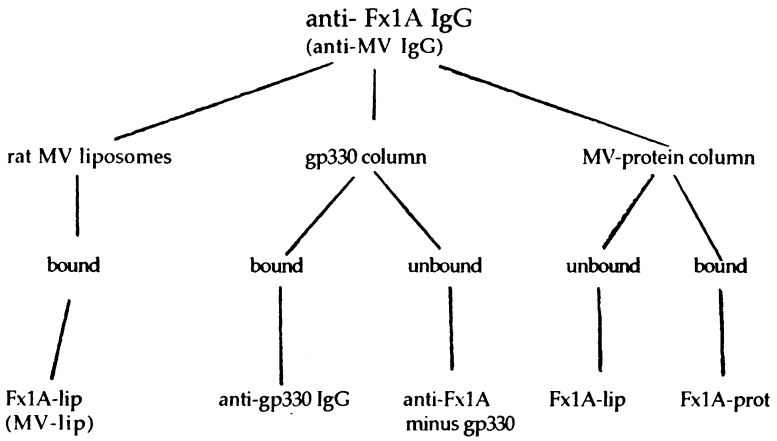

Figure 1. *Fractionation of antisera to Fx1A and microvilli.*

neutralized with 1 mol/L Tris HCl, pH 8, dialyzed against PBS at 4 C for 24 hours and concentrated with Aquacide II.

Sheep anti-Fx1A IgG and rabbit anti-MV IgG were depleted of antibodies specific for microvillar proteins by affinity absorption with delipidated isolated microvillar proteins. The delipidated protein residue that remained as a by-product of the liposome preparation was dried with a stream of N_2 and dissolved in 2% SDS (Bio-Rad Laboratories, Richmond, CA), diluted to a final concentration of 0.2% of SDS with 0.1 mol/L $NaHCO_3$, and coupled to CNBr-Sepharose 4B. The IgGs were circulated over this column, and the bound IgG (designated anti-Fx1A-prot or anti-MV-prot IgG) was eluted with 100 mmol/L glycine-HCl buffer, pH 2.7. The absorption was repeated 10 to 15 times, until no antibody against any microvillar or renal cortical protein was detected by immunoblotting. The IgG fractions depleted of activities against protein antigens obtained were designated anti-Fx1A-lip IgG or anti-MV-lip IgG. The fractionation procedure is summarized in Figure 1.

Immunoblotting on Microvillar Proteins

Isolated microvilli were dissolved in SDS sample buffer (7.2% SDS, 9 mmol/L EDTA, 20% glycerol, 10 mmol/L dithiothreitol, 13 mmol/L Tris-phosphate buffer, pH 6.8) by boiling for 3 minutes. The proteins were separated by 7% SDS-PAGE[6] and transferred onto nitrocellulose.[16] Immune overlays were performed by incubation of transfers with the following antibodies: sheep anti-Fx1A IgG (1 µg/ml); anti-Fx1A-lip IgG from sheep or anti-MV-lip IgG from rabbit (150 µg/ml); anti-Fx1A-prot (4 µg/ml); anti-Fx1A IgG minus gp330 (4 µg/ml); affinity-purified anti-gp330 IgG (1 µg/ml); affinity-purified anti-DPP IV IgG (1 µg/ml). These antibodies were detected with alkaline phosphatase-labeled affinity-purified goat anti-rabbit IgG (Promega, Madison, WI), or alkaline phosphatase-labeled rabbit anti-sheep IgG and 5-bromo-4-chloro-3-indolylphosphate and nitrobluetetrazolium in 100 mmol/L Tris-HCl buffer, pH 9.5 (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Immunoblotting of Glycolipids

Antibody binding to rat kidney glycolipids was detected by a modified method of Magnani et al.[17] Briefly, extracted microvillar glycolipids were separated by chromatography on a glass-backed thin-layer chromatography plate (Merck Kieselgel 60 WF, Darmstadt, Germany), using chloroform/methanol/water (60/35/8) as running solution. Some plates were stained with orcinol. Plates were dried and soaked in 4% solution of poly-isobutyl-metacrylate (Aldrich, Steinheim, Germany) in hexane for 90 seconds. After air-drying, the plates were incubated in 3% BSA-PBS and the chromatograms were overlaid in 3% BSA-PBS for 1 hour at 20 C with the following antibodies: sheep (data not shown) and rabbit anti-MV IgG; rabbit anti-gp330 IgG; nonimmune rabbit IgG. All IgGs were used at a dilution of 1:200. The plates were washed with PBS, and bound antibody was detected by sequential incubations with biotinylated anti-rabbit Ig, streptavidin peroxidase (Amersham, Braunschweig, Germany), and the peroxidase substrate 4-chloronaphtole.

Induction of Heymann Nephritis

Rats were injected in the tail vein with 200 to 600 µg IgG in 500 µl PBS. The amount of IgG used in the different experimental groups is given in Tables 1 and 2. The 24-hour urine of the animals was collected from days 4 to 7 in metabolic cages, and proteinuria was measured by the biuret method.[18] Animals were sacrificed on day 7, and kidneys were flushed blood-free with ice-cold PBS and snap-frozen in N_2-cooled isopentane. For immunoelectron microscopy, the kidneys were fixed by perfusion with paraformaldehyde-lysine-periodate solution (PLP), as described.[19]

Immunohistochemistry

Immunofluorescence was performed on unfixed 3-µ cryostat sections or on 1-µ frozen sections of PLP-fixed kidneys, cut on a Reichert Ultracut ultramicrotome, equipped with an F4 cryo-stage, as described.[20] Immune deposits were detected by direct

Table 1. *Proteinuria and Activation of C5b-9 MAC by Antibodies to Fx1A and MV Injected in Rats*

Antibody specific for	IgG injected (mg)/200 g rat	Proteinuria (mg/24 hr)	Immune deposits contain	
			IgG	MAC
Fx1A*	4.5	6.2	Sheep	+
	6.7	5.5	Sheep	+
	8.9	8.7	Sheep	+
	11.2	60.0	Sheep	++
Affi-gp330*	0.17	8.1	Sheep	0
	0.70	3.0	Sheep	0
	1.70	5.9	Sheep	0
Fx1A minus gp330*	0.54	2.3	0	0
Rat MV†	10.5	47.3	Rabbit	++
PBS	0	8.7	0	0

* Sheep IgG.
† Rabbit IgG.

Table 2. *Proteinuria and Activation of C5b-9 MAC by Antibodies to gp330 and Lipid Fractions Injected in Rats*

Antibody specific for	IgG injected (mg)/200 g rat	Proteinuria (mg/24 hr)	Immune deposits contain	
			IgG	MAC
Fx1A-prot*	0.49	7.6	Sheep	0
	0.58	4.9	Sheep	0
	0.70	3.3	Sheep	0
Fx1A-lip*	0.55	3.0	0	0
MV-lip†	0.40	5.2	0	0
	1.57	3.0	0	0
Fx1A-lip* plus affi-gp330*	0.17/0.24	10.9	Sheep	+
	0.55/0.47	3.2	Sheep	+
MV-lip† plus affi-gp330*	0.40/0.15	5.7	Sheep + rabbit	+
	1.57/1,7	31.3	Sheep + rabbit	++
PBS	0	8.7	0	0

* Sheep IgG.
† Rabbit IgG.

immunofluorescence with FITC-conjugated rabbit anti-goat $F(ab)_2$ fragment (Jaxell Accurate Chemicals, Westbury, NY), which was found to react also with sheep IgG and which was depleted of cross-reactivity by preabsorbtion with rat IgG and rabbit IgG bound to CNBr-agarose (Sigma Chemical Co., St. Louis, MO) or with FITC-mouse anti-rabbit $F(ab)_2$, which was preabsorbed with sheep IgG and rat IgG bound to CNBr-Sepharose. The C5b-9 (MAC) neoantigen was localized by indirect immunofluorescence using a monoclonal antibody obtained from Dr. W. G. Couser, as described,[8] and as second antibody goat anti-mouse $F(ab)_2$ (Jaxell) which was preabsorbed on sheep, rabbit, and rat IgG bound to CNBr-agarose (Sigma). For negative controls the first antibodies were omitted or replaced by irrelevant mouse IgG.

Localization of Fx1A-lip and C5b-9 on ultrathin frozen sections was performed as described.[20] Briefly, PLP-fixed kidneys of rats that were injected with sheep anti-gp330 IgG plus rabbit anti-MV-lip IgG (group 1), or normal rat kidneys (group 2), were cut on a Reichert Ultracut ultramicrotome equipped with a F4 cryo-stage. The sections in group 1 were incubated with 10-nm gold particles conjugated to sheep anti-rabbit IgG (diluted 1:50; Auroprobe, Amersham). Other sections were incubated with monoclonal anti-C5b-9 IgG[8] followed by a bridging rabbit anti-mouse IgG and 10-nm gold anti-rabbit IgG conjugate. Sections in group 2 were incubated in rabbit anti-MV-lip IgG (10 μg/ml), followed by 10-nm gold anti-rabbit IgG conjugate. The sections were processed as described[20] and examined in a JEOL 1200 electron microscope.

Injected antibodies to laminin and DPP IV were localized by direct immunoperoxidase electron microscopy. Briefly, sections of PLP-fixed rat kidneys that were injected with affinity-purified sheep anti-gp330 IgG (420 μg, 530 μg), followed by rabbit antibodies to laminin (750 μg IgG) or to DPP IV (450 μg IgG), were incubated with sheep anti-rabbit IgG F(ab)′ fragments or with rabbit anti-sheep IgG F(ab)′ fragments, conjugated to horseradish peroxidase (diluted 1:100, Biosys, Compiegne, France), and processed as described.[7,20,21]

Results

Subfractions of Anti-Fx1A IgG

Sheep anti-Fx1A IgG and rabbit anti-MV IgG showed similar properties in their polyspecific pattern of binding to microvillar proteins (Figure 2) and to glycolipids (Figure 3) in immunoblots. They also induced granular subepithelial immune deposits after intravenous injection (Figure 4A), activated complement C5b-9 membrane attack complex (Figure 4B), and produced heavy proteinuria (Table 1).

When anti-Fx1A or anti-MV IgGs were absorbed with protein-free liposomes prepared from isolated microvilli, rapid agglutination reaction was observed that was only partially reversed when the bound IgG was eluted with alkaline elution buffer. The yield of the IgG released from the liposomes amounted to ~0.2% of the initial anti-Fx1A or anti-MV IgG. These anti-Fx1A or anti-MV-lip IgG fractions failed to label any microvillar protein by immunoblotting (Figure 2).

As an alternative approach for the purification of the lipid-specific anti-Fx1A-lip or anti-MV-lip fractions, the polyspecific anti-Fx1A or anti-MV IgGs were completely depleted of antibodies with specificity for microvillar proteins by repeated absorption to an affinity column containing delipidated microvillar proteins (Figure 2). This non-protein-specific IgG obtained

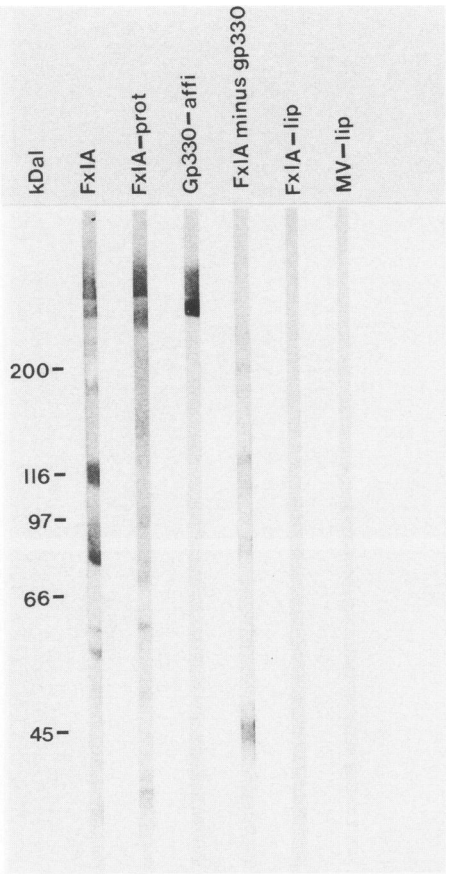

Figure 2. *Characterization of antibody fractions used for injection into rats by immunoblotting on rat microvillar proteins (separated by 3.6–10% SDS-PAGE). Antibodies used: sheep anti-Fx1A IgG, anti-Fx1A-prot IgG, obtained from the binding fraction of anti-Fx1A IgG to an affinity column containing a preparation of delipidated rat microvillar proteins; gp330-affi, affinity-purified sheep antibodies to gp330; Fx1A minus gp330 IgG, obtained after depletion of antibodies directed against gp330; Fx1A-lip IgG, obtained after absorbing the anti-Fx1A IgG or anti-MV IgG proteins to protein-free liposomes of rat tubular microvilli or after removal of all antibodies directed against microvillar proteins. Both anti-glycolipid fractions fail to recognize microvillar proteins, even when the anti-MV-lip IgG was used in a very high concentration for incubation of the nitrocellulose transfers (150 μg/ml).*

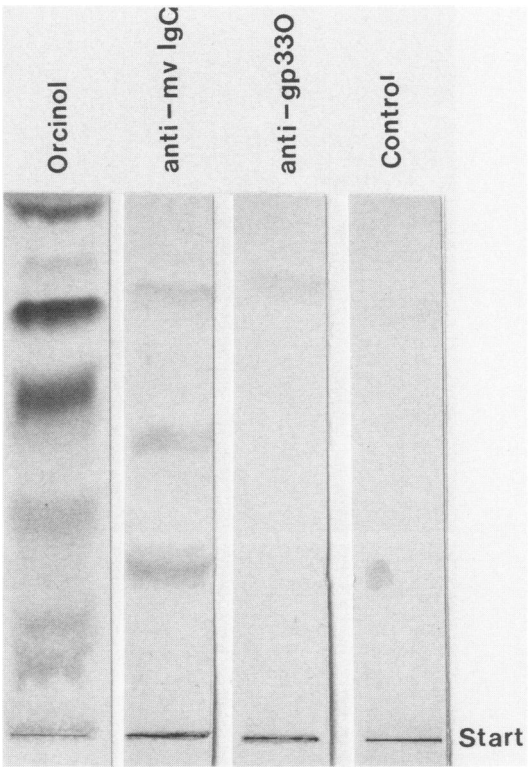

Figure 3. *Microvillar glycolipids, as resolved by thin-layer chromatography of a glycolipid extract prepared by suspending isolated rat microvillar membrane fractions in apolar solvents. Orcinol staining reveals several major glycolipid bands, while immunoblotting with anti-MV IgG shows a more restricted pattern which, however, clearly indicates the multispecificity of this antibody for several glycolipids. Affinity-purified anti-gp330 IgG as well as a nonimmune rabbit serum control fails to bind to any glycolipid.*

showed identical properties to that purified by absorption onto liposomes. Both IgGs failed to bind to glomeruli after intravenous injection (Figure 5E).

The IgG fraction designated anti-Fx1A-prot was specific for microvillar proteins (Figure 2) but devoid of any glycolipid-binding IgG. This IgG fraction immunostained proximal tubular brush borders (data not shown) and produced formation of immune deposits after injection, but it failed to activate complement (Figure 5, C and D) and did not cause proteinuria (Table 2).

When the antibody fractions anti-MV-lip and Fx1A-prot were recombined and injected into rats, granular immune deposits as well as C5b-9 activation and proteinuria were observed (data not shown) which was expected.

Affinity-Purified Anti-gp330 IgG Induces Immune Deposits

Affinity-purified anti-gp330 IgG was prepared from the polyspecific anti-Fx1A and anti-MV IgGs and was found to label exclusively gp330 by immunoblotting (Figure 2) and the basal aspect of the microvillar brush borders by immunohistochemistry. This IgG induced granular immune deposits 7 days after injection (Figure 5C), as described[8]; however, it failed to activate complement (Figure 5D) or induce proteinuria (Table 1). Immunoblots on microvillar glycolipid fractions showed no specific binding (Figure 3).

When the anti-Fx1A IgG or anti-MV IgG was selectively and completely depleted of IgG specific for gp330 to yield the fraction anti-Fx1A minus gp330 IgG, it was found to label the brush border region of proximal tubules by immunofluorescence; however, it

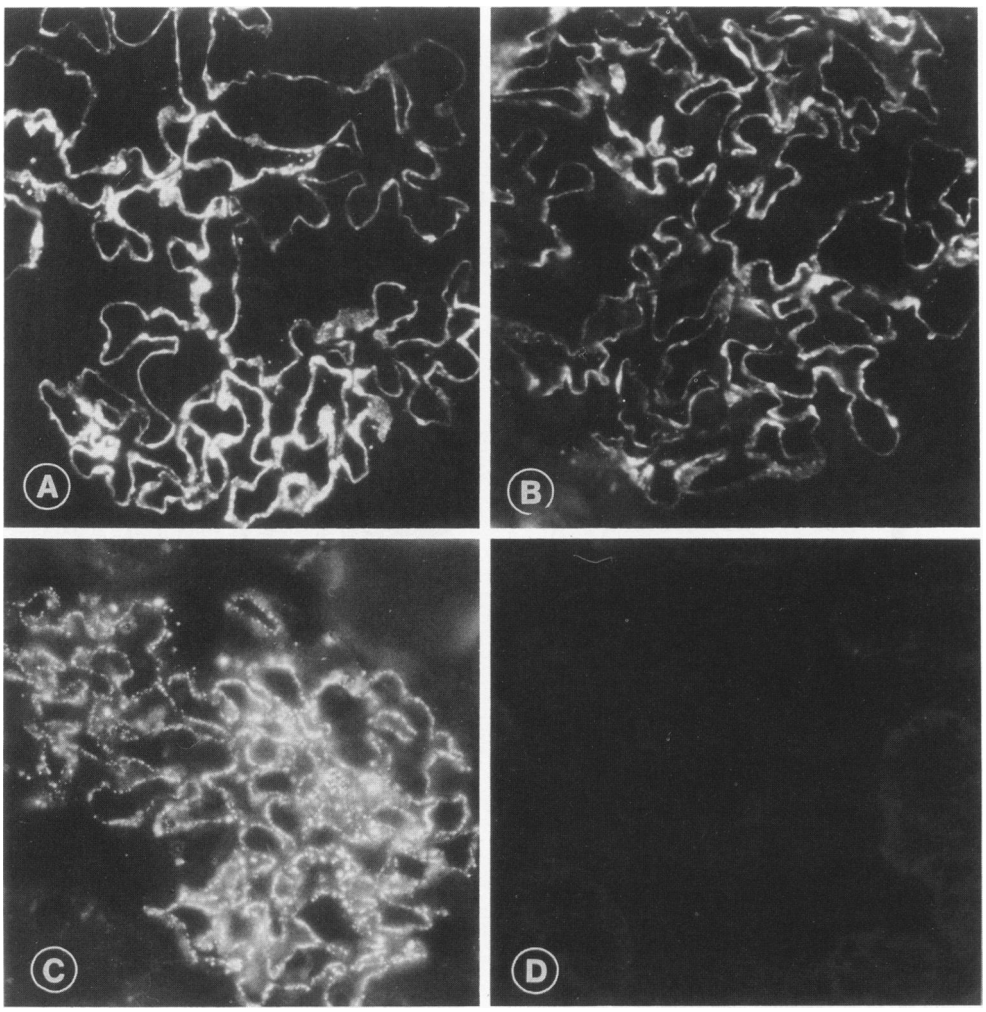

Figure 4. *Comparative localization by immunofluorescence of IgG and of C5b-9 in glomeruli 6 days after injection of unfractionated anti-Fx1A IgG* (**A, B**) *and the Fx1A-prot IgG fraction* (**C, D**) **A:** *Granular pattern of sheep IgG within the glomerular immune deposits;* **B:** *Deposition of the C5b-9 complement complex.* **C** *and* **D:** *Injection of the Fx1A-prot IgG directed against microvillar proteins.* **C:** *Sheep antibodies within the immune deposits similar to* **A,** *but with no activation of the complement C5b-9 complex, as shown in* **D** (×600).

failed to bind to glomeruli and to activate complement after injection (Figure 5, E and F).

Glycolipid-Specific IgG Fractions Activate Complement and Induce Proteinuria

To determine directly whether the lipid-specific IgG fractions contain complement-activating antibodies, we combined for injection into rats affinity-purified anti-gp330 IgG (which induced immune deposits but failed to activate complement) with anti-MV-lip IgG, which by itself does not bind to glomeruli after intravenous injection. The combination of the antibodies resulted in the formation of immune deposits (Figure 5A) and activation of the C5b-9 complement membrane attack complex (Figure 5B). While no proteinuria was observed in animals when small amounts of IgG were injected (400 μg anti-MV-lip IgG and 150 μg anti-gp330 IgG), overt proteinuria of >30 mg/day developed when this amount was increased to 1.57 mg anti-MV-lip IgG and 1.7 mg anti-gp330 IgG (Table 2). C5b-9 neoantigen was detected also in the immune deposits in rats injected with low doses of antibodies and which did not develop proteinuria (Figure 5B).

The glomerular localization of the individual injected IgGs was revealed when sheep anti-gp330 IgG was combined with rabbit anti-MV-lip-IgG, or conversely. Both sheep and rabbit IgGs were then detected within the immune deposits by direct immunofluorescence in these mixing experiments. In addition, we confirmed this localization by cryo-immunoelectron microscopy, where it was found that the injected rabbit anti-MV-lip IgG localized within the immune deposits (Figure 6, A and B).

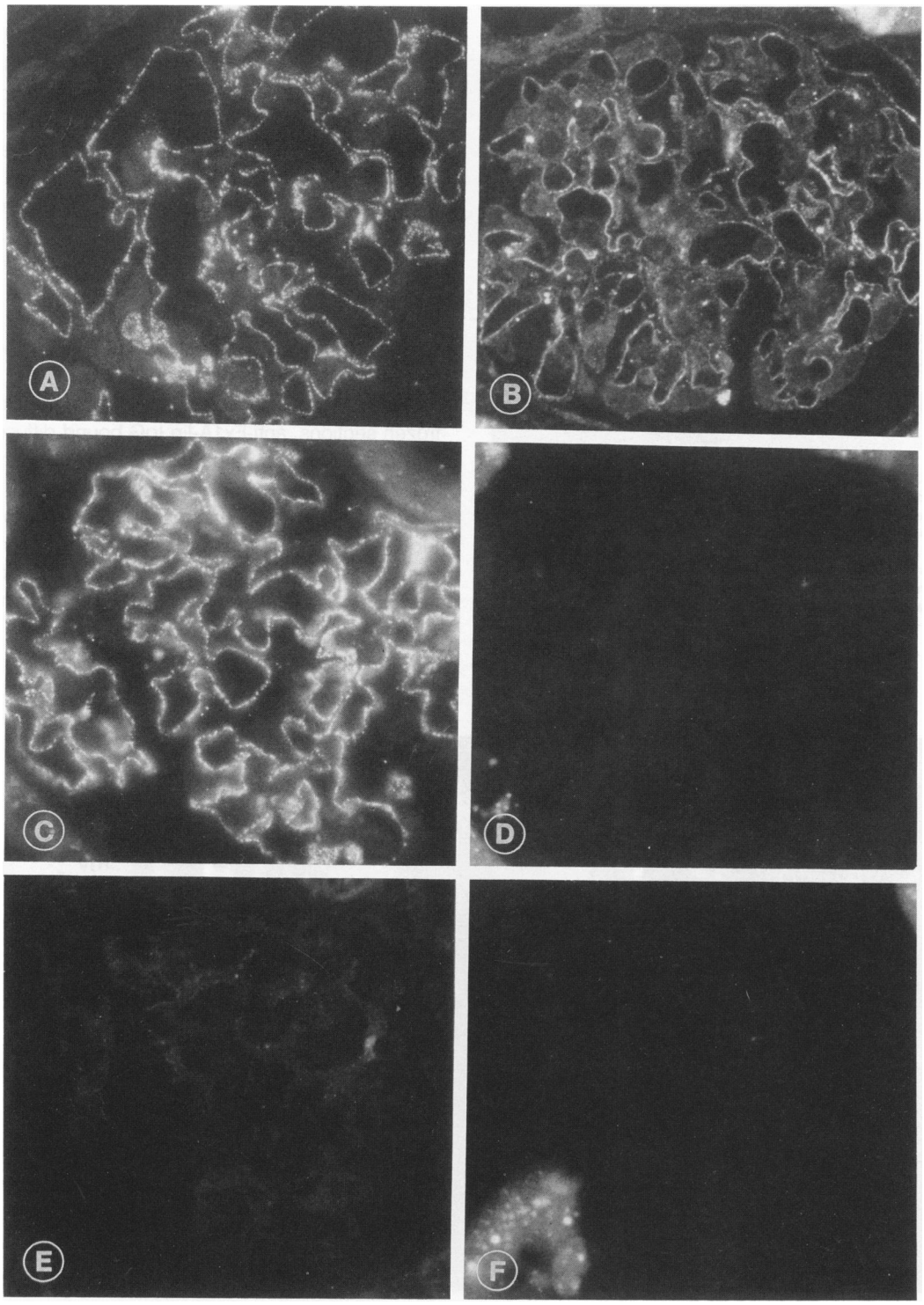

Figure 5. *Comparative localization of IgG and C5b-9 in glomeruli 6 days after injection of antibody fractions prepared from anti-Fx1A IgG by immunofluorescence. The localization of IgG is shown in* A, C, *and* E, *and that of C5b-9 in* B, D, *and* F. *Antibodies used for injection:* A *and* B: *A mixture of affinity-purified sheep anti-affi-gp330 IgG plus rabbit anti-MV-lip IgG.* C *and* D: *Affinity-purified sheep anti-gp330 IgG alone.* E *and* F: *Rabbit anti-MV-lip IgG alone. These experiments indicate that immune deposits are formed by antibodies to gp330* (A, C), *while C5b-9 deposition requires also antibodies specific for microvillar glycolipids* (B) (×600).

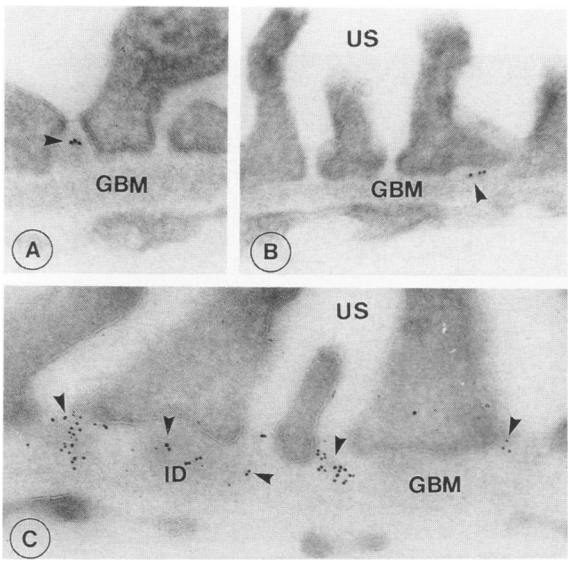

Figure 6. *Localization by immunogold electron microscopy on ultrathin frozen sections of anti-MV-lip IgG* (**A, B**) *and of C5b-9* (**C**) *in a mixing experiment, in which affinity-purified sheep anti-gp330 IgG and rabbit anti-MV-lip IgG were combined for injection. Gold particles in* **A** *and* **B** *indicate the localization of rabbit anti-MV-lip IgG which is found in immune deposits* (arrowheads). **C**: *C5b-9 is also found within immune deposits (ID) in a similar preparation. US, urinary space; GBM, glomerular basement membrane (×32,000).*

Localization of the Fx1A-lip Glycolipid Antigen(s)

The brush borders of proximal tubules of normal rat kidneys were intensely labeled by anti-Fx1A-lip IgG by immunofluorescence on cryostat sections (Figure 7A). In addition, the basolateral aspects of the epithelial cells of proximal tubules and of cortical collecting ducts were stained (Figure 7A). Glomeruli showed a very light, diffuse staining. The binding of anti-Fx1A-lip antibody was completely abolished when the kidney sections were extracted with chloroform, methanol, or ethanol before incubation, while the immunolabeling with anti-gp330 IgG was not affected (data not shown).

By immunogold electron microscopy on ultrathin frozen sections, anti-Fx1A-lip IgG bound diffusely to the surface membranes of glomerular epithelial, endothelial, and, to a lesser extent, mesangial cells (Figure 8). Coated pits were not labeled. In proximal tubules, the membranes of brush border microvilli, intermicrovillar domains, apical vesicles, and basolateral membranes were labeled (Figure 7, B and C).

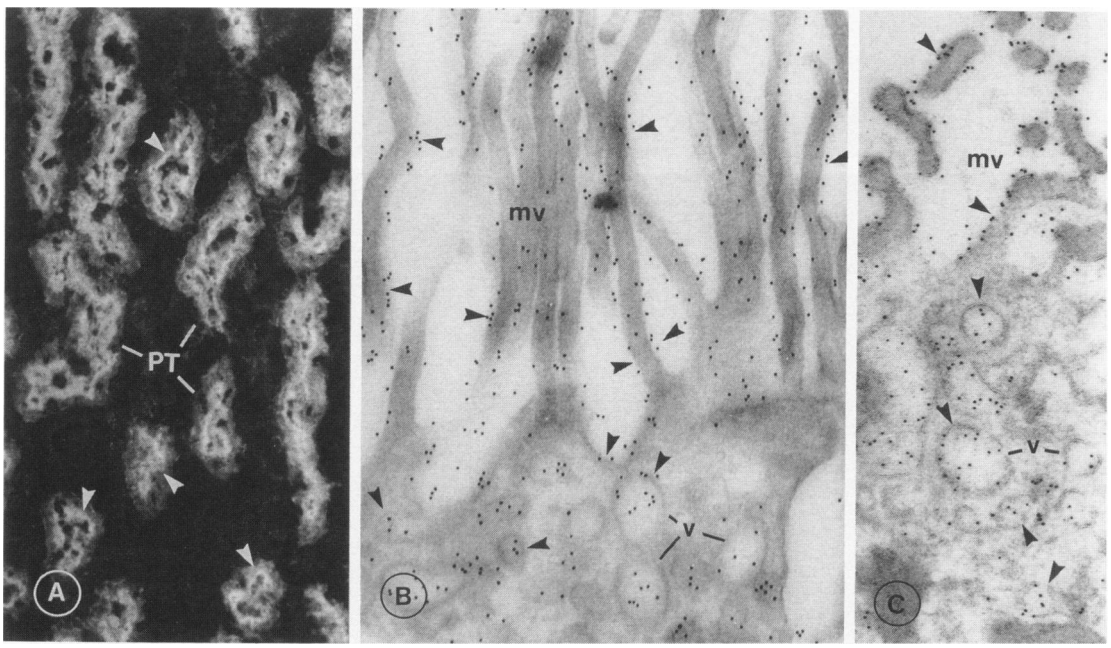

Figure 7. *Localization of the Fx1A-lip antigen (s) by immunofluorescence and on ultrathin frozen section with lipid-specific anti-MV-lip antibodies eluted from microvillar liposomes prepared from the glycolipids of tubular microvilli.* **A**: *Intense labeling by indirect immunofluorescence of the brush border of proximal tubules (PT) is seen. Faint staining of the basolateral regions of tubules is indicated by arrowheads.* **B** *and* **C**: *Indirect immunogold labeling on ultrathin frozen sections of proximal tubules shows labeling of the membranes of brush border microvilli* (**arrowheads,** *MV), of the intermicrovillar membrane domains, and of the apical vesicles (V). By contrast to gp330, there is no specific labeling of coated pits (×32,000).*

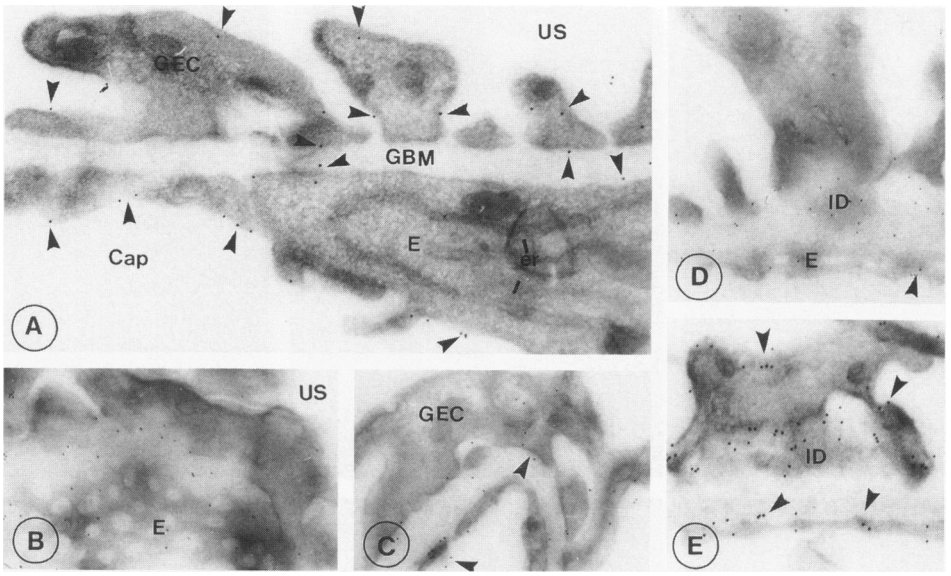

Figure 8. *Localization of the Fx1A-lip antigen (s) on ultrathin frozen sections in glomeruli of normal rats* (**A-C**) *and in PHN 6 days after injection of anti-Fx1A IgG* (**D** *and* **E**). *In all figures, the cell membranes of glomerular epithelial cells (GEC) and endothelial cells* (**E**) *are labeled by gold particles* (**arrowheads**). *There is no obvious polarity, and the glycolipid antigen (s) are evenly distributed on the surfaces facing the urinary space (US), the glomerular basement membrane (GBM), and the capillary lumen (CAP).* **D** *and* **E**: *In 6-day PHN gold particles also label the immune deposits (ID)* (×*32,000*).

Gold particles were only rarely found in the glomerular basement membrane (GBM) or on the membranes of intracellular organelles (Figure 8C); they were observed, however, within the matrix of immune deposits of rats 7 days after injection of sheep anti-Fx1A IgG (Figure 8, D and E).

Injection of Anti-gp330 IgG Mixed with Anti-Laminin and Anti-DPP IV IgG

Previous reports have suggested a role in PHN for antibodies directed against DPP IV in the development of proteinuria[4,21–23] and against laminin.[24] We have therefore mixed affinity-purified sheep anti-gp330 IgG (from sheep anti-Fx1A IgG) with rabbit antibodies specific for rat DPP IV or laminin. When injected into rats, these mixtures of antibodies consistently failed to activate complement or induce proteinuria (Figure 9, C and F), although both IgGs were detected by immunofluorescence in glomeruli from days 1 to 3 after injection (Figure 9, A, B, D, and E). In addition, we found by immunoelectron microscopy that 7 days after injection, sheep anti-gp330 IgG was exclusively present within the immune deposits, while the antibodies to DPP IV were not (Figure 10).

Discussion

The intraglomerular activation of complement to the C5b-9 membrane attack complex is essential for the development of proteinuria in PHN[8–10]; however, the molecular mechanisms for this process are not known. Polyspecific antisera raised against crude kidney cortex fractions, such as Fx1A, RTEα5, or isolated rat microvilli, are able to induce full-blown PHN with immune deposits and severe proteinuria 5 to 7 days after injection.[22] By contrast, intravenous injection of monospecific anti-gp330-HNAC antibodies prepared by affinity chromatography readily produced subepithelial immune deposits but failed to activate complement or induce proteinuria.[23] The simplest interpretation of these findings is that in addition to the gp330-HNAC-dependent mechanisms of immune deposit formation, there is at least one additional antibody species within the polyspecific antisera raised against Fx1A or rat MV that is responsible for the local activation of complement in PHN. Previous results from other laboratories, as well as experiments in this report, have not supported the concept that complement activation and C5b-9 formation are mediated by antibodies to glomerular proteins, such as the enzyme DPP IV.[21,23,24] This has raised the intriguing question as to whether a subfraction of C5b-9-activating and proteinuria-inducing antibodies is present within the polyspecific antisera to Fx1A or MV which involves glycolipid rather than protein antigen(s).

We have designed a strategy to separate the nephritogenic polyvalent anti-Fx1A and anti-MV IgGs

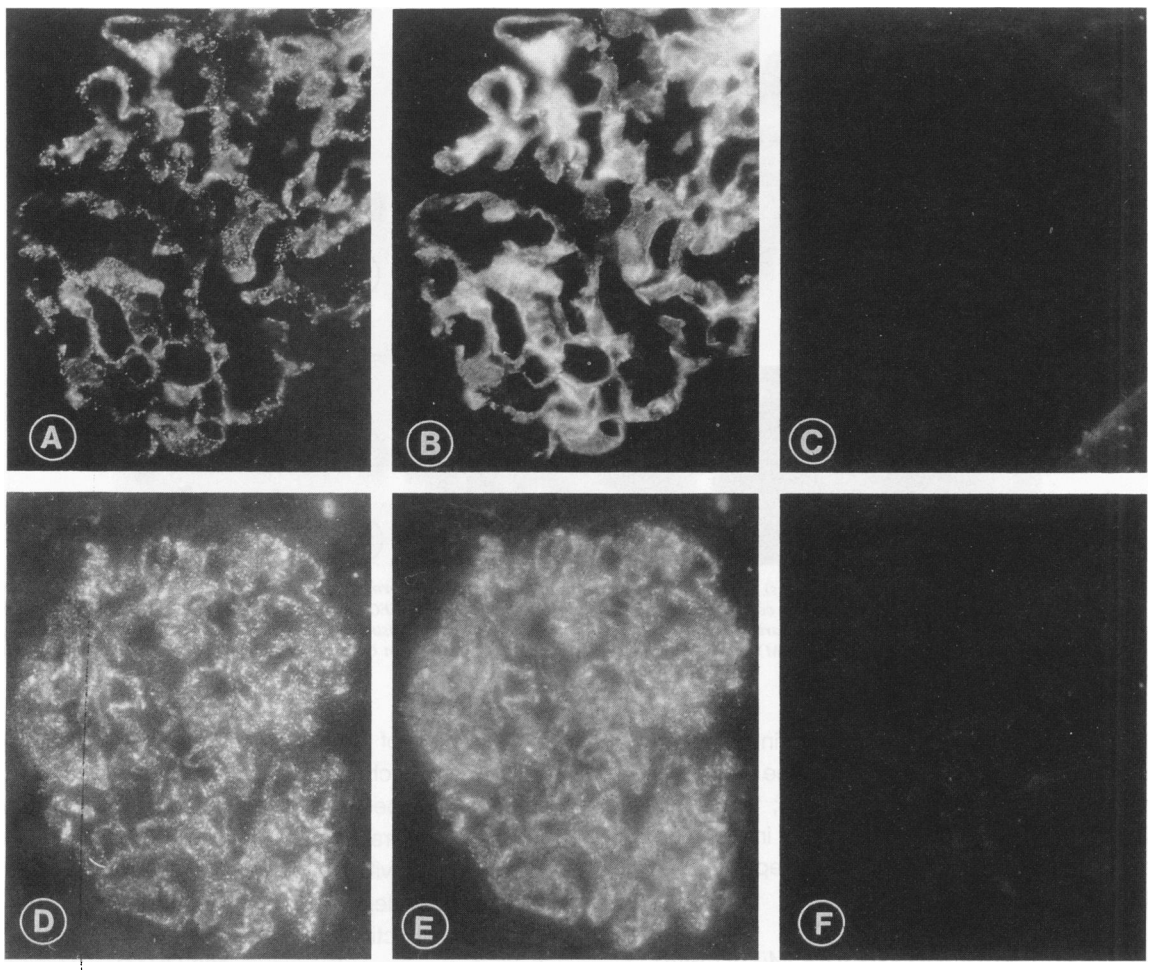

Figure 9. *Localization of antibodies to gp330, laminin, DPP IV, and C5b-9 in a mixing experiment in which sheep anti-gp330 IgG plus rabbit anti-laminin IgG* (**A, B, C**) *and sheep anti-gp330 IgG plus rabbit anti-DPP IV IgG* (**D, E, F**) *were injected and the kidneys fixed 3 days after injection. Sheep anti-gp330 IgG* (**A, D**)*, rabbit anti-laminin IgG* (**B**)*, and rabbit anti-DPP IV IgG* (**E**) *are present within the glomerular loops by immunofluorescence, but the complement C5b-9 complex* (**C, F**) *is not activated* (×600).

into two fractions by immunoabsorption, yielding one with exclusive specificity for the glycolipids of rat microvilli (Fx1A-lip) and another directed against microvillar proteins (Fx1A-prot). To test for their ability to activate complement and induce proteinuria, these IgG fractions were injected into rats either separately or in combination. The rat kidneys were evaluated by immunohistochemistry for the formation of glomerular immune deposits and the presence of C5b-9 membrane attack complex. These approaches have provided evidence that the glycolipid-specific IgG fraction is responsible for the *in situ* activation of complement with formation of C5b-9, and, at least in part, also for the development of proteinuria. This conclusion is supported by the finding that injection of a mixture of IgGs specific for gp330-HNAC and for the MV-lip or Fx1A-lip fractions induce immune deposits and also activate C5b-9 membrane attack complex of complement, while the individual antibody preparations fail to do so. The glycolipid-specific fractions were found to be equally active when prepared either by affinity purification on protein-free microvillar liposomes or when the protein-specific antibodies were completely depleted from polyspecific anti-Fx1A or anti-MV IgGs by absorption onto isolated microvillar proteins. When the amount of the injected glycolipid-specific antibodies in combination with affi-gp330 IgG exceeded a threshold of ~0.75 mg IgG/100 g rat (Table 2), overt proteinuria was observed, which was up to >80% of that observed when PHN was induced by an equivalent amount of unfractionated anti-Fx1A IgG. Proteinuria developed under these conditions even though the most avidly binding anti-glycolipid antibodies were obviously not eluted from the agglutinated liposomes used for immunoabsorption. These findings agree with previous observations in which a threshold amount of glomerular antibody was found to be necessary to induce proteinuria in PHN.[25] It further

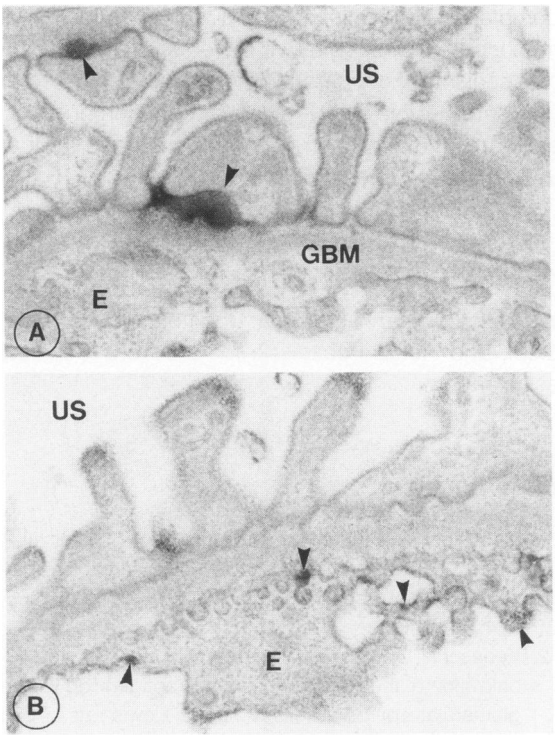

Figure 10. *Localization of affinity-purified sheep anti-gp330 IgG* (**A**) *and anti-DPP IV IgG* (**B**) *in a mixing experiment in which sheep anti-gp330 IgG plus rabbit anti-DPP IV IgG were injected on day 6 before sacrifice. Sheep anti-gp330 IgG was found within the immune deposits* (**arrowheads**) *by a direct immunoperoxidase method, but anti-DPP IV antibodies were absent and were only found on endothelial cell surfaces. US, urinary space; E, endothelial cells; GBM, glomerular basement membrane.* Arrowheads *indicate membrane vesicles* (×25,000).

appears that the Fx1A-prot IgG fraction contains the potency to produce immune deposits after intravenous injection, presumably because it contains, among other specificities, the IgG directed against gp330-HNAC. By contrast, this fraction has failed completely to activate complement in our hands. Since the Fx1A-prot IgG fraction contains the entire repertoire of antibodies to microvillar proteins and lacks those specific for glycolipids, this provides further evidence that the complement-activating principle is contained within the glycolipid-specific fraction.

As indicated by immunoelectron microscopy, there is apparently a close association of the gp330-HNAC immune complexes and the glycolipid antigens. This is supported by the observation that antibodies to Fx1A-lip or MV-lip do not bind to glomeruli and do not activate complement when injected alone. However, they co-localize with anti-gp330-HNAC IgG within the immune deposits when co-injected with anti-gp330-HNAC IgG. Obviously, the species specificity of the IgG is not of relevance, because in some mixing experiments sheep antibody to gp330 and rabbit IgG to MV-lip or vice versa were used with the same results.

This raises the question of whether the microvillar glycolipids are also present within the normal glomerulus and could serve there as a target for their specific antibodies to form immune complexes *in situ,* similar to the role of gp330-HNAC in the formation of immune deposits.[7,24]. When anti-MV-lip IgG was used for the localization of glycolipids, they were found along the cell membranes of podocytes, but not in clathrin-coated pits where gp330 is located. The glycolipid antigen(s) were also localized on the surface membranes of glomerular endothelial cells. However, these data do not provide a definite localization of the glycolipid antigen(s) which induce complement activation upon antibody binding, because of the presumable polyspecificity of the currently available anti-MV-lip antibodies. This raises the possibility that the glycolipids found within the immune deposits are derived from endogenous glomerular cells rather than from circulating immune complexes, although definite evidence for this mechanism is lacking.

It is at present not known by which molecular mechanisms antibodies to glomerular glycolipids could activate or assist in the activation of complement at the levels of podocyte cell membranes and/or within the immune deposits. There are several examples in which glycolipids influence *in vitro* interactions of components of the alternate pathway, especially of C3 and factor H. For example, phosphatidylethanolamine binds with high-affinity human C3, and modifications of phosphatidylethanolamine amino groups were shown to decrease the binding activity of factor H to C3b.[26] Furthermore, modifications of sialic acid residues of gangliosides (especially of those on the C-9 carbon of the polyhydroxylated tail and on the carboxyl group) were shown to greatly reduce the inhibitory effect of sialic acids on the alternate pathway by reduction of binding of factor H to C3b.[27,28] In PHN, one hypothetical explanation could be that the immune complex formation of the anti-glycolipid antibodies occurs via carbohydrate side chains and may thus modify the basal cell surface of the podocyte to become an acceptor for complement components and subsequently influence their interaction, for example, by reducing the binding affinity of factor H to C3.

Recently a role for macrophages in the development of proteinuria in PHN was suggested by the observation that their average number of ~5 in normal controls was increased in proteinuric rats to >20 per glomerulus. Depletion of macrophages was shown to delay the onset of proteinuria.[29] While there is no con-

ceivable role for macrophages in the activation of complement in PHN, it is possible that their number per glomerulus increases after this event. Our search with a panel of monoclonal marker antibodies on cryostat sections, however, has failed to show a significant increase in macrophages, thus making it unlikely, but not impossible, that they could contribute to glomerular damage (unpublished observations).

The cellular mechanisms that convert the formation of C5b-9 and its insertion into the cell membranes of podocytes[9] to damage of the glomerular filter and proteinuria are not completely understood at present. One hypothesis is that C5b-9 induces the synthesis of the enzymes of the NADPH-oxido-reductase complex, which is externalized by podocytes and subsequently generates locally reactive oxygen species that could modify matrix proteins of the GBM.[30] This is plausible, because the expression of the key enzyme of the oxido-reductase complex, cytochrome b_{558} in glomeruli in PHN, was found to be suppressed by complement depletion with cobra venom factor.[30] Thus, the activation of complement by immune complex formation with glycolipid antigen(s) could initiate this cascade of events, which are associated with the development of proteinuria.

The results of this study appear to rule out the possibility that the enzyme DPP IV[31,32] or laminin[33] serve as possible antigen(s) for the complement-activating "second immune complex system of PHN." These proteins were suspected, because it was observed that antibodies eluted from glomeruli of rats with PHN induced by polyspecific antibodies occasionally immunoblotted DPP IV and laminin, in addition to gp330.[29,31,33] However, it appears that antibodies to these proteins only slightly enhance glomerular damage when injected together with anti-gp330 IgG.[24,33,34] In addition, the results presented in this paper show that anti-DPP IV and anti-laminin antibodies do not enter the immune deposits at all when injected together with anti-gp330-HNAC IgG. Taken together, these and other data[34] suggest that anti-DPP IV and anti-laminin IgG play little, if any, role in the development of proteinuria in PHN.

Collectively, these results indicate that nephritogenic antibodies raised against the crude kidney cortex glycolipoprotein preparation Fx1A and against isolated tubular microvilli contain two groups of specificity: one that is responsible for the formation of subepithelial immune deposits that was identified as the gp330-HNAC[6], and a second fraction that contains the complement-activating IgG and which is present in the glycolipid-specific fraction. This latter IgG binds only to glomeruli in cooperation with gp330-HNAC immune complexes. The precise identification of the complement-activating glycolipid and production of monospecific antibodies will be required to clarify the mechanisms of cooperation of the anti-gp330-HNAC IgG and the role of the glycolipid antigen-antibody system in the activation of complement and the development of proteinuria in PHN.

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